Wan Ahmad Kamil, W. N. I., Takebayashi, Y., Findlay, J., Heesom, K., & Avison, M. (2018). Impact of OqxR loss of function on the envelope proteome of Klebsiella pneumoniae and susceptibility to antimicrobials. *Journal of Antimicrobial Chemotherapy*, *73*(11), 2990-2996. [dky293]. https://doi.org/10.1093/jac/dky293

Impact of OqxR loss of function on the envelope proteome of *Klebsiella pneumoniae* and susceptibility to antimicrobials.

Wan Ahmad Kamil WAN NUR ISMAH,[1,2] Yuiko TAKEBAYASHI,[1] Jacqueline FINDLAY,[1] Kate J. HEESOM,[3] and Matthew B. AVISON[1*].

[1]School of Cellular & Molecular Medicine, University of Bristol, Bristol. UK

[2]Faculty of Biotechnology & Biomolecular Sciences, Universiti Putra Malaysia, Selangor Darul Ehsan, Malaysia

[3]Bristol Proteomics Facility, University of Bristol, UK

***Correspondence: Matthew B. Avison, School of Cellular & Molecular Medicine, Biomedical Sciences Building, University Walk, Bristol. BS81TD. UK. bimba@bris.ac.uk. +44(0)1173312063.**

Running Title: OqxR loss in *K. pneumoniae*

Abstract

OBJECTIVES

In *Klebsiella pneumoniae*, loss of function mutations in the transcriptional repressors RamR and OqxR both have an impact on the production of efflux pumps and porins relevant to antimicrobial efflux/entry. Our aim was to define, in an otherwise isogenic background, the relative effects of OqxR and RamR loss of function mutations on envelope protein production, envelope permeability, and antimicrobial susceptibility. We have also investigated the clinical relevance of an OqxR loss of function mutation, particularly in the context of β-lactam susceptibility.

METHODS

Envelope permeability was estimated using a fluorescent dye accumulation assay. Antimicrobial susceptibility was measured using disc testing. Total envelope protein production was quantified using LC-MS/MS proteomics, and quantitative RT-PCR was used to measure transcript levels.

RESULTS

Loss of RamR or OqxR reduced envelope permeability in *K. pneumoniae* by 45-55% relative to wild-type. RamR loss activated AcrAB efflux pump production ≈5-fold, and this reduced β-lactam susceptibility, conferring ertapenem non-susceptibility even in the absence of a carbapenemase. In contrast, OqxR loss specifically activated OqxAB efflux pump production >10,000-fold. This reduced fluoroquinolone susceptibility but had little impact on β-lactam susceptibility even in the presence of a β-lactamase.

CONCLUSIONS

Whilst OqxR loss and RamR loss are both seen in *K. pneumoniae* clinical isolates, only RamR loss significantly stimulates AcrAB efflux pump production. This means that only RamR mutants have significantly reduced β-lactamase mediated β-lactam susceptibility and therefore represent a greater clinical threat.

Introduction

Multiple envelope proteins contribute to antimicrobial susceptibility and resistance in *Klebsiella pneumoniae*. Two major porins, OmpK35 and OmpK36 are important for drug entry,[1–4] and the OmpK35/36 ratio is affected by osmolarity.[5] Drug efflux is mainly catalysed by the Resistance Nodulation Cell Division (RND)-type pump, AcrB, working with AcrA and TolC as a tripartite system.[6–8] AcrAB production is controlled by the transcriptional repressor AcrR and is upregulated following overproduction of the AraC-type transcriptional activator RamA.[6,9,10] RamA overproduction also boosts transcription of *micF*, encoding an antisense RNA, which downregulates OmpK35 porin production, and RamA upregulates a second RND-type efflux pump, OqxAB.[10] RamA hyper-production occurs in *ramR* loss of function mutants because RamR is a transcriptional repressor of *ramA*.[9-11] Loss of function mutations in the transcriptional repressor OqxR also activate *oqxAB* transcription, as well as transcription of *rarA*, which encodes a RamA orthologue.[11,12] To add to this complexity, increased efflux pump and/or reduced porin production can confer an additive effect in combination with plasmid mediated antimicrobial resistance proteins or target site mutations. This has been demonstrated for the complex interplay of factors that influence ciprofloxacin susceptibility,[13] and in the generation of potentially important and perhaps clinically under-reported carbapenemase negative carbapenem resistance phenotypes.[10,14]

We have previously characterise the interplay between factors that confer RamA mediated multi-drug resistance in *K. pneumoniae*.[10] The aim of the work reported here was to analyse the importance of *oqxR* loss of function mutation in multi-drug resistance, and to identify the resistance proteins involved.

Materials and Methods

Bacterial strains and susceptibility testing

Four K. pneumoniae strains were used in the study. Ecl8,[15] plus *in vitro* selected loss of function mutants in *oqxR* (Tyr109STOP) or *ramR* (Thr124Pro)[13] and finally, Ecl8Δ*ramR* [16] additionally carrying a *gyrA* QRDR (Ser83Phe) and an *oqxR* loss of function mutation (Arg93Cys).[13] *E. coli* XL10-Gold (Stratagene) was used for cloning. Disc susceptibility testing

was performed according to CLSI methodology [17] and interpreted using CLSI performance standards.[18]

Cloning genes, transformation and complementation studies

The wild-type *oqxR* and *ramR* genes of *K. pneumoniae* Ecl8 were amplified by PCR using the primers listed in **Table S1**. Each PCR amplicon was TA cloned into pCR2.1-TOPO (Invitrogen) according to the manufacturer's instructions. The inserts were removed using EcoRI (for *oqxR*) or HindIII plus XbaI (for *ramR*) and ligated into pK18.[19] The *rarA* gene was PCR amplified from Ecl8 using primers listed in **Table S1** and directly ligated into pBAD322K [20] after cutting with NcoI and XbaI. Cloning of plasmid-mediated β-lactamase genes $bla_{\text{CTX-M-1}}$ and $bla_{\text{CMY-4}}$ (with their native promoters) using the vector pSU18 [10] and later sub-cloning into the vector pUBYT has previously been reported.[14] Recombinant plasmids were used to transform *K. pneumoniae* strains to kanamycin (30 mg/L for pBAD322 and 50mg/L for pUBYT) using electroporation. All strains and plasmids are recorded in **Table S2**.

Quantitative analysis of envelope proteome via Orbitrap LC-MS/MS and quantitative RT-PCR

Each mutant and transformant was cultured in 50 mL Cation Adjusted Mueller-Hinton broth (MHB) (Sigma) with appropriate antibiotic selection. Cultures were incubated with shaking (160 rpm) at 37°C until the optical density at 600 nm reached 0.5-0.7. Total cellular RNA was purified and qRT-PCR was performed as previously described,[10,21] using the primers listed in **Table S1**. Total envelope proteomics was performed as previously described.[10,21] For each LC-MS/MS proteomics experiment, raw protein abundance data were collected for three biological replicates of each test condition. A paired T-test was used to calculate the significance of any difference in protein abundance in pooled data from the two test conditions. A *p*-value of <0.05 was considered significant. The fold change in abundance for each protein in the two test conditions was calculated using the averages of absolute abundances for the three biological replicates for the two test conditions.

Fluorescent Hoechst (H) 33342 dye accumulation assay

Envelope permeability was estimated as described previously [21] in bacteria grown in MHB using an established fluorescent dye accumulation assay.[22] The assay was performed using a

black, flat-bottomed 96-well plates (Greiner Bio-one, Stonehouse, UK) and a Fluostar Optima (Aylesbury, UK) plate reader. H33342 dye (Sigma) was used at a final concentration of 2.5 µM.

Results and Discussion

Relative impact of OqxR and RamR loss of function mutations on antimicrobial susceptibility and envelope permeability

We have recently reported *in vitro* selection of *oqxR* and *ramR* loss of function mutants from the wild-type *K. pneumoniae* strain Ecl8.[13] **Table 1** reports the relative impacts of these mutations on inhibition zone diameter for a range of antimicrobials of different classes. As expected, the *ramR* point mutant phenotype was very similar to the profile seen for an Ecl8Δ*ramR* mutant previously studied.[10] The *oqxR* mutation had a larger effect on fluoroquinolone and chloramphenicol susceptibility than the *ramR* mutation (68 mm versus 47 mm combined zone diameter reduction across five agents); the *ramR* mutation had a larger impact on cephalosporin susceptibility than the *oqxR* mutation (69 mm versus 46 mm combined zone diameter reduction across 10 agents). Both mutations conferred minocycline non-susceptibility though neither had any effect on the zone diameters around carbapenem or aminoglycoside discs.

Overall envelope permeability (a combination of outer membrane permeability and efflux) in the two mutants was similar. The steady state accumulation of a fluorescent dye was ≈45% and ≈55% of wild-type for the *ramR* and *oqxR* mutants, respectively (**Fig. 1**).

Efflux pump and porin protein abundance changes in oqxR and ramR mutants

We used Orbitrap LC-MS/MS proteomics to define the impact of the *ramR* and *oqxR* mutations on the production of porins and efflux pumps previously implicated in antimicrobial drug resistance in *K. pneumoniae*. The OqxA and OqxB efflux pump proteins were found to be below the detection level in wild-type cells, but they were detectable in both the *ramR* and *oqxR* mutants, with upregulation being almost two orders of magnitude greater in the latter (**Fig. 2c,d**). By contrast, upregulation of AcrA and AcrB efflux pump proteins was only observed in the *ramR* mutant (**Fig. 2a,b**) with similar fold increases to those reported recently for an Ecl8Δ*ramR* mutant (4.79- versus 3.88-fold for AcrA and 3.56- versus

4.87-fold for AcrB).[10] Whist it has not been confirmed experimentally in *K. pneumoniae*, TolC is likely to work as the outer membrane protein for both OqxAB and AcrAB. TolC levels were significantly upregulated in both *oqxR* and *ramR* mutants relative to wild type (**Fig. 2e**). Downregulation of the OmpK35 porin was equally observed in both mutants: 0.43-fold, p=0.005 (*oqxR* mutant), 0.41-fold p=0.005 (*ramR* mutant) (**Fig. 2f**). Significant changes in abundance of the OmpK36 porin were not observed in either mutant (**Fig. 2g)**. A list of proteins for which abundance was significantly changed (≥2-fold, p<0.05) in the *oqxR* or *ramR* mutant relative to Ecl8 during growth in MHB are shown in **Table S3** and **Table S4**. Ten of eleven proteins previously shown to be significantly up-regulated ≥2-fold upon deletion of *ramR* in Ecl8, irrespective of growth conditions,[10] were also regulated in the *ramR* point mutant; the only protein significantly down-regulated ≥2-fold in Ecl8Δ*ramR* and the Ecl8 *ramR* point mutant was OmpK35 (**Table S4**). Twelve of 23 proteins differentially regulated in the *oqxR* loss of function mutant (**Table S3**) during growth in MHB were also differentially regulated in the *ramR* mutant (**Table S4**) when grown in the same conditions.

To confirm the relative impacts of the *oqxR* and *ramR* mutations on efflux pump and porin production, we took a previously selected *oqxR*/*ramR* double mutant,[13] and individually complemented either *oqxR* or *ramR in trans*. The double mutant produced levels of AcrAB similar to the *ramR* mutant and levels of OqxAB similar the *oqxR* mutant (**Fig. 3 a,b,c,d**; **Fig. 2 a,b,c,d**). We found TolC or OmpK35 production to be maximally altered in the *oqxR* or *ramR* single mutants because protein levels were similar in the *oqxR*/*ramR* double mutant to levels in the *oqxR* or *ramR* single mutants (**Fig. 3 e,f,g; Fig. 2 e,f,g**). However, the additive effect of the two regulatory mutations on AcrB/OqxB efflux pump protein production was enough to further reduce envelope permeability to ≈25% of wild-type, according to the fluorescent dye accumulation assay (**Fig. S1**). This is because, in protein abundance terms, AcrB and OqxB are limiting (**Fig. 2**). Complementation of either *ramR* or *oqxR* in the double mutant increased envelope permeability to ≈55% of wild-type (**Fig. S1**), the level of permeability seen in the single mutants (**Fig. 1**), confirming that there are no other significant factors involved. Likewise, complementation of the double mutant with either *oqxR* or *ramR* resulted in a recombinant that produced efflux pump and porin proteins as expected given the remaining mutation (**Fig. 3 a,b,c,d**; **Fig. 2 a,b,c,d**).

Impact of RarA overproduction in the oqxR mutant

We have shown that *oqxR* loss has a large impact on OqxAB but no significant (p>0.05) impact on AcrAB abundance (**Fig. 2**). This would fit with OqxR being a local transcriptional repressor of *oqxAB*, as previously demonstrated.[11,12] However, loss of *oqxR* significantly upregulates TolC and reduces levels of OmpK35 (**Fig. 2**). It has previously been shown that OqxR controls transcription of *rarA*,[12] encoding a RamA-like global transcriptional activator.[23] Accordingly, we hypothesised that the wider effects of *oqxR* loss in Ecl8 were caused by overexpression of *rarA*. qRT-PCR revealed that *rarA* expression is indeed upregulated (417 ± 127-fold) in the *oqxR* mutant. Expression of *acrA* does not significantly change (1.4 ± 0.1-fold) in the *oqxR* mutant, as predicted from the proteomics (**Fig. 2**).

In order to dissect out the implications of RarA overproduction on the proteomic changes seen in the *oqxR* mutant, we cloned *rarA* without its promoter into pBAD322K [20] and set about overexpressing it by using arabinose induction. qRT-PCR confirmed *rarA* expression in Ecl8(pBAD322K:*rarA*) in the presence of 0.02% w/v arabinose was very similar (415 ± 87.2 fold more than control) to that seen in the *oqxR* mutant. Key efflux and porin protein production levels following *rarA* overexpression at this level **(Fig. S2)** were very similar to those seen in the *ramR* loss of function mutant **(Fig. 2)** in which *ramA*, a close homologue of *rarA*, is overexpressed.[10]

Based on the phenotype of the Ecl8 *oqxR* mutant (**Fig. 2**), the very different impact of *rarA* overexpression on protein levels in Ecl8 (**Fig. S2**) was surprising. Whilst *rarA* expression was the same in the *oqxR* loss of function mutant and in the *rarA* over-expressing, *oqxR* wild-type recombinant (see above), increased AcrAB production was only seen in the *rarA* over-expressing, *oqxR* wild-type recombinant (**Fig 2, S2**). The positive impact of *rarA* overexpression on *acrAB* expression in an *oqxR* wild-type background has also been reported by others.[12] We hypothesise that RarA preferentially binds to the *oqxAB* promoter, so when OqxR is not present – in the *oqxR* mutant – the overproduced RarA predominantly drives *oqxAB* expression, and there is little impact on *acrAB* expression. However, when RarA is overproduced in a background when OqxR is located as a repressor on the *oqxAB* promoter (an artificial situation unlikely ever to be seen in a clinical isolate) RarA binds at less preferential sites, including the *acrAB* promoter.

Impact of OqxR loss of function in combination with plasmid mediated cephalosporinases on carbapenem susceptiblity

We have recently shown that *ramR* loss of function enhances the impact of carrying the serine active site cephalosporinases $bla_{CTX\text{-}M\text{-}1}$ and $bla_{CMY\text{-}4}$, with particularly worrying effects on carbapenem susceptibility, including causing ertapenem resistance in some cases.[10,14] To see whether OqxR loss has a similar impact, plasmids encoding $bla_{CTX\text{-}M\text{-}1}$ or $bla_{CMY\text{-}4}$, were used to transform our Ecl8 *oqxR* point mutant. There was a very slight impact on ertapenem susceptibility in comparison with Ecl8 transformants (**Table S5**), but much less than that seen previously in an Ecl8Δ*ramR* mutant.[10] This probably reflects the relatively low impact of *oqxR* loss on β-lactam susceptibility (**Table 1**), because of its negligible impact on AcrAB efflux pump production (**Fig. 2**) and the fact that OqxAB is not a noted β-lactam efflux pump.[24]

PCR sequencing was used to identify *oqxR* mutations in a collection of 44 *K. pneumoniae* clinical isolates where *ramR* sequence and β-lactamase complement had already been determined.[10] All *oqxR* mutants were confirmed to hyper-produce OqxAB using LC-MS/MS proteomics of whole cell extracts as previously described.[13] As expected from our work with Ecl8 transformants (**Table S5**), there was no impact of the *oqxR* loss of function mutation on carbapenem susceptibility in isolates M and AD, which carry $bla_{CTX\text{-}M\text{-}15}$, though, as previously noted, loss of *ramR* causes ertapenem resistance in $bla_{CTX\text{-}M\text{-}15}$ carrying isolate T (**Table S6**).[10]

Conclusion

It is known that *oqxR* loss of function causes *oqxAB* over-expression, and this is associated with tigecycline resistance.[12,25,26] It is also known that *rarA* is overexpressed, and that when overproduced in a recombinant form, RarA can have a wide range of effects on the cell, including upregulation of *acrAB* and *tolC* transcription.[21,23] However, our use of proteomics has allowed the first analysis of the relative impact of these events on the abundance of efflux pump and porin proteins in *K. pneumoniae* and has allowed us to define the relative importance of *oqxR* and *ramR* loss of function mutations, which can both be found in clinical *K. pneumoniae* isolates.[13] Loss of OqxR has a dramatic effect on OqxAB efflux pump production, with the absolute abundance of OqxB in an *oqxR* mutant being similar to that of AcrB (≈2 x10^9 units) (**Fig. 2**). The impact of this >10,000-fold upregulation of OqxAB on antimicrobial susceptibility is rather modest, however (**Table 1**). There is strong effect on fluoroquinolone susceptibility, conferring ciprofloxacin resistance in the presence of other mechanisms.[13] OqxAB overproduction also causes chloramphenicol, minocycline and, reportedly, tigecycline resistance (**Table 1**),[25,26] but its overall impact on β-lactam

susceptibility is low, even in the presence of a plasmid mediated cephalosporinase (**Table 1, S5, S6**). This limits the clinical importance of *oqxR* loss relative to *ramR* loss and is likely to be because there is a negligible effect of *oqxR* loss on AcrAB production (**Fig. 2**). Indeed, when AcrAB is overproduced only ≈5-fold more than wild-type levels, as seen in a *ramR* mutant, this confers ertapenem resistance in the presence of a cephalosporinase.[10] Our work here also adds support to our hypothesis that OmpK35 downregulation is not particularly important for reducing β-lactam susceptiblity,[10] since OqxR loss and RamR loss both cause a similar downregulation of OmpK35 (**Fig. 2**) but very different effects on β-lactam susceptibility (**Table 1, S5, S6**).

We were slightly surprised that *oqxR* loss does not cause AcrAB upregulation, because it has previously been shown that *oqxR* loss causes *rarA* over-expression,[12] and that RarA overproduction from a recombinant system in an *oqxR* wild-type background causes AcrAB upregulation.[12,23] Whilst we were able to reproduce these previous findings (**Fig. S2**), the key here is that RarA overproduction in an OqxR wild-type background, as used previously to define the *rarA* regulon [23] is not relevant to what happens in an *oqxR* loss of function mutant. The impact of RarA overproduction is less broad in an *oqxR* mutant because it predominantly acts as a positive feed-forward mechanism to dramatically activate *oqxAB* transcription, explaining the >10,000-fold upregulation of OqxAB production seen (**Fig. 2**) rather than taking on a highly pleotropic role. Nonetheless, RarA overproduction does have additional effects in the cell, even when overproduced due to *oqxR* loss, and many of the genes controlled are also controlled by RamA (**Table S3**).

Funding

This work was funded by grant MR/N013646/1 to M.B.A. and K.J.H. and grant NE/N01961X/1 to MBA from the Antimicrobial Resistance Cross Council Initiative supported by the seven research councils. W. A. K. W. N. I. was funded by a postgraduate scholarship from the Malaysian Ministry of Education.

Transparency Declaration

None to declare – All authors.

References

1. Albertí S, Rodríquez-Quiñones F, Schirmer T, *et al*. A porin from *Klebsiella pneumoniae*: sequence homology, three-dimensional model, and complement binding. *Infect Immun* 1995; **63**: 903–10.

2. Doménech-Sánchez A, Martínez-Martínez L, Hernández-Allés S, *et al*. Role of *Klebsiella pneumoniae* OmpK35 porin in antimicrobial resistance. *Antimicrob Agents Chemother* 2003; **47**: 3332–5.

3. Sugawara E, Kojima S, Nikaido H. *Klebsiella pneumoniae* Major Porins OmpK35 and OmpK36 Allow More Efficient Diffusion of β-Lactams than Their *Escherichia coli* Homologs OmpF and OmpC DiRita VJ, ed. *J Bacteriol* 2016; **198**: 3200–8.

4. Hernández-Allés S, Albertí S, Rubires X, *et al*. Isolation of FC3-11, a bacteriophage specific for the *Klebsiella pneumoniae* porin OmpK36, and its use for the isolation of porin-deficient mutants. *Can J Microbiol* 1995; **41**: 399–406.

5. Tsai Y-K, Fung C-P, Lin J-C, *et al*. *Klebsiella pneumoniae* outer membrane porins OmpK35 and OmpK36 play roles in both antimicrobial resistance and virulence. *Antimicrob Agents Chemother* 2011; **55**: 1485–93.

6. Padilla E, Llobet E, Doménech-Sánchez A, *et al*. *Klebsiella pneumoniae* AcrAB efflux pump contributes to antimicrobial resistance and virulence. *Antimicrob Agents Chemother* 2010; **54**: 177–83.

7. Nikaido H. Multidrug efflux pumps of gram-negative bacteria. *J Bacteriol* 1996; **178**: 5853–9.

8. Nikaido H, Zgurskaya HI. AcrAB and related multidrug efflux pumps of *Escherichia coli*. *J Mol Microbiol Biotechnol* 2001; **3**: 215–8.

9. Schneiders T, Amyes SGB, Levy SB. Role of AcrR and RamA in Fluoroquinolone Resistance in Clinical *Klebsiella pneumoniae* Isolates from Singapore. *Antimicrob Agents Chemother* 2003; **47**: 2831–7.

10. Jiménez-Castellanos J-C, Wan Nur Ismah WAK, Takebayashi Y, *et al*. Envelope proteome changes driven by RamA overproduction in *Klebsiella pneumoniae* that enhance acquired β-lactam resistance. *J Antimicrob Chemother* 2018; **73**: 88-94.

11. Bialek-Davenet S, Lavigne J-P, Guyot K, *et al*. Differential contribution of AcrAB and OqxAB efflux pumps to multidrug resistance and virulence in *Klebsiella pneumoniae*. *J Antimicrob Chemother* 2015; **70**: 81–8.

12. Veleba M, Higgins PG, Gonzalez G, *et al*. Characterization of RarA, a novel AraC family multidrug resistance regulator in *Klebsiella pneumoniae*. *Antimicrob Agents Chemother* 2012; **56**: 4450–8.

13. Wan Nur Ismah WAK, Takebayashi Y, Findlay J, *et al*. Prediction of fluoroquinolone susceptibility directly from whole genome sequence data using liquid chromatography-tandem mass spectrometry to identify mutant genotypes. *Antimicrob Agents Chemother* 2018; **62**: e01814-17

14. Takebayashi Y, Wan Nur Ismah WAK, Findlay J, *et al*. Prediction Of Cephalosporin And Carbapenem Susceptibility In Multi-Drug Resistant Gram-Negative Bacteria Using Liquid Chromatography-Tandem Mass Spectrometry. *BioRxiv* 2017: 138594.

15. George AM, Hall RM, Stokes HW. Multidrug resistance in *Klebsiella pneumoniae*: a novel gene, *ramA*, confers a multidrug resistance phenotype in *Escherichia coli*. *Microbiology* 1995; **141**: 1909–20.

16. De Majumdar S, Yu J, Spencer J, *et al*. Molecular basis of non-mutational derepression of *ramA* in *Klebsiella pneumoniae*. *J Antimicrob Chemother* 2014; **69**: 2681–9.

17. Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Disk Susceptibility Tests – Ninth Edition: Approved Standard M2-A9. CLSI, Wayne, PA, USA. 2006.

18. Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Susceptibility Testing: Twenty-fourth Informational Supplement M100-S24. CLSI, Wayne, PA, USA. 2014.

19. Pridmore RD. New and versatile cloning vectors with kanamycin-resistance marker. *Gene*

1987; 56:309-12.

20. Cronan JE. A family of arabinose-inducible *Escherichia coli* expression vectors having pBR322 copy control. *Plasmid* 2006; **55**: 152–7.

21. Jiménez-Castellanos J-C, Wan Ahmad Kamil WNI, Cheung CHP, *et al*. Comparative effects of overproducing the AraC-type transcriptional regulators MarA, SoxS, RarA and RamA on antimicrobial drug susceptibility in *Klebsiella pneumoniae*. *J Antimicrob Chemother* 2016; **71**: 1820-5.

22. Coldham NG, Webber M, Woodward MJ, *et al*. A 96-well plate fluorescence assay for assessment of cellular permeability and active efflux in *Salmonella enterica* serovar Typhimurium and *Escherichia coli*. *J Antimicrob Chemother* 2010; **65**: 1655–63.

23. De Majumdar S, Veleba M, Finn S, *et al*. Elucidating the regulon of multidrug resistance regulator RarA in *Klebsiella pneumoniae*. *Antimicrob Agents Chemother* 2013; **57**: 1603–9.

24. Rodríguez-Martínez JM, Díaz de Alba P, Briales A, *et al*. Contribution of OqxAB efflux pumps to quinolone resistance in extended-spectrum-β-lactamase-producing *Klebsiella pneumoniae*. *J Antimicrob Chemother* 2012; **68**: 68–73.

25. Zhong X, Xu H, Chen D, *et al*. First Emergence of acrAB and oqxAB Mediated Tigecycline Resistance in Clinical Isolates of *Klebsiella pneumoniae* Pre-Dating the Use of Tigecycline in a Chinese Hospital Zhang Q, ed. *PLoS One* 2014; **9**: e115185.

26. Sheng Z-K, Hu F, Wang W, *et al*. Mechanisms of Tigecycline Resistance among *Klebsiella pneumoniae* Clinical Isolates. *Antimicrob Agents Chemother* 2014; **58**: 6982-5.

Table 1: Disc susceptibility testing for *K. pneumoniae* Ecl8 and its *ramR* and *oqxR* mutants

Antibiotic disc (µg in disc)	Diameter of growth inhibition zone (mm) around antibiotic disc, or difference in zone diameter relative to control		
	Ecl8	*ramR*	*oqxR*
Ciprofloxacin (5)	37	-12	-15
Norfloxacin (10)	33	-9	-14
Ofloxacin (5)	31	-9	-11
Levofloxacin (5)	33	-8	-10
Chloramphenicol (30)	29	-9	-18
Cefotaxime (30)	37	-7	-4
Ceftizoxime (30)	36	-4	-2
Cephalothin (30)	25	-8	-5
Cefoxitin (30)	33	-13	-11
Cefotetan (30)	29	-3	NC
Cefuroxime (30)	30	-11	-9
Ceftazidime (30)	35	-7	-5
Ceftriaxone (30)	35	-6	-5
Cefoperazone (75)	32	-7	-5
Cefepime (30)	32	-3	NC
Aztreonam (30)	35	-3	-3
Imipenem (10)	27	NC	NC
Meropenem (10)	30	NC	NC
Doripenem (10)	27	NC	NC
Ertapenem (10)	29	NC	NC
Minocycline (30)	22	-10	-7
Tetracycline (30)	26	-6	-5
Tigecycline (15)	20	-5	-3
Amikacin (30)	25	NC	NC
Gentamicin (10)	23	NC	NC
Tobramycin (10)	23	NC	NC
Kanamycin (30)	23	NC	NC

Values reported are the means of three repetitions rounded to the nearest integer. Zone diameters are reported for Ecl8; difference from the Ecl8 values are reported for the *ramR* or *oqxR* loss of function mutants. NC means no change in zone diameter relative to Ecl8. Shading represents non-susceptibility (resistance or intermediate resistance) according to breakpoints set by the CLSI.[18]

335 **Figure Legends**

336

337 **Figure 1: The accumulation of H33342 dye over a 30 cycle (45 minute) incubation period by**
338 ***K. pneumoniae oqxR* and *ramR* mutants relative to Ecl8 wild-type.** In each case, fluorescence
339 of mutant cells incubated with the dye is presented relative to wild type Ecl8 cells (set to
340 100%) after each cycle. Each line shows mean data for three biological replicates with 8
341 technical replicates in each. Error bars define the standard error of the mean.

342

343 **Figure 2: Abundance of key envelope proteins in *K. pneumoniae* Ecl8 wild-type and the *oqxR***
344 **and *ramR* mutants measured using LC-MS/MS proteomics.** Values reported are absolute
345 abundance values ($x10^{-7}$, arbitrary units) recorded by the instrument used. Data are means
346 (n=3 biological replicates) error bars are standard error of the mean. Stars (*) above a bar
347 indicate a significant difference in abundance relative to control based on the following rules:
348 fold difference in abundance is $\geq$ 2 and $p<0.05$ for a T-test comparing absolute protein
349 abundance data, n=3.

350

351 **Figure 3: Envelope proteome changes in complemented mutants; Δ*ramR*+*oqxR*::pk18(*oqxR*)**
352 **and Δ*ram*R+*oqxR*::pk18(*ramR*) in comparison with plasmid-only control.** Values reported
353 are absolute abundance values ($x10^{-7}$, arbitrary units) recorded by the instrument used. Data
354 are means (n=3 biological replicates) error bars are standard error of the mean. Stars (*)
355 above a bar indicate a significant difference in abundance relative to control based on the
356 following rules: fold difference in abundance is $\geq$ 2 and $p<0.05$ for a T-test comparing absolute
357 protein abundance data, n=3.

358

359

360

Figure 1

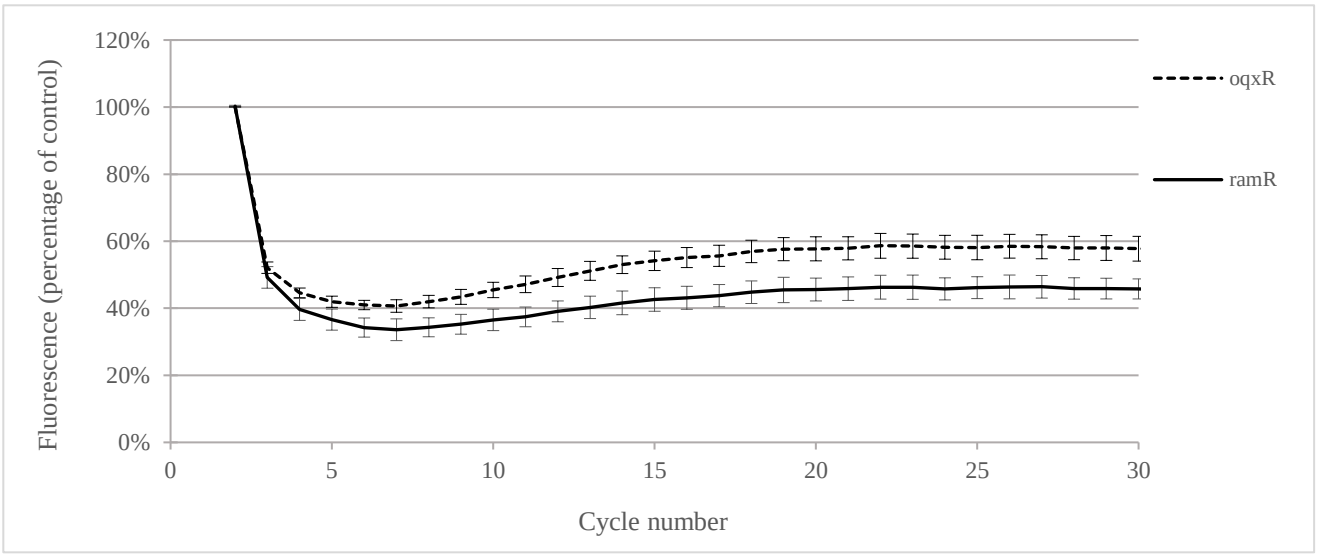

120%
100%
80%
60%
40%
20%
0%
Fluorescence (percentage of control)
0
5
10
15
20
25
30
Cycle number
oqxR
ramR

Figure 2.

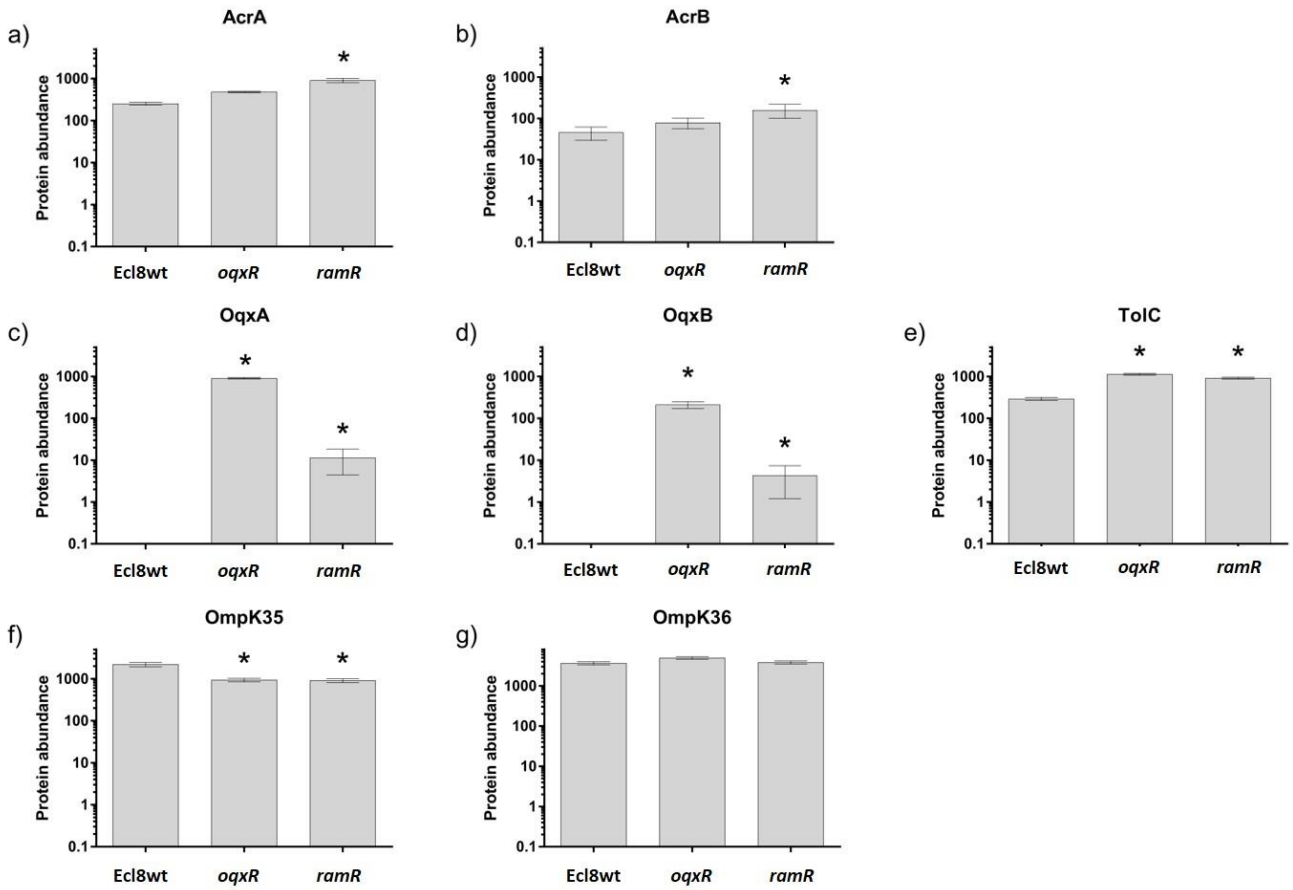
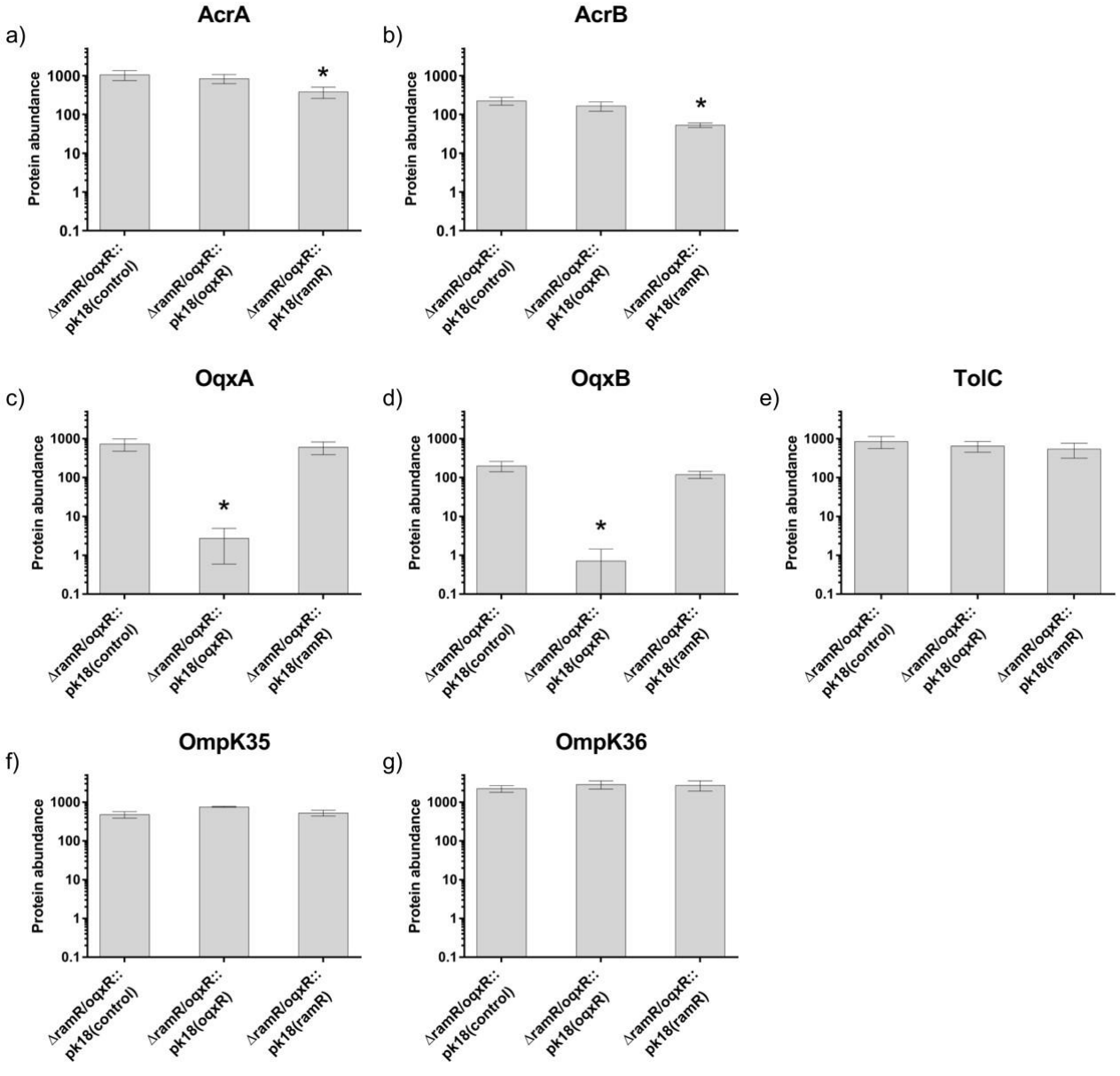

a)
AcrA
b)
AcrB
c)
OqxA
d)
OqxB
e)
TolC
f)
OmpK35
g)
OmpK36
Protein abundance
1000
100
10
1
0.1
*
Ecl8wt
oqxR
ramR

Figure 3.


a)
AcrA
b)
AcrB
c)
OqxA
d)
OqxB
e)
TolC
f)
OmpK35
g)
OmpK36
Protein abundance
1000
100
10
1
0.1
*
ΔramR/oqxR::pk18(control)
ΔramR/oqxR::pk18(oqxR)
ΔramR/oqxR::pk18(ramR)